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Saturation site-directed mutagenesis of thymidylate synthase.
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A Lactococcus lactis gene encodes a membrane protein with putative ATPase activity that is homologous to the essential Escherichia coli ftsH gene product.
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A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli ftsH* gene product

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A gene, encoding a protein homologous to an essential *Escherichia coli* protein, FtsH, was identified adjacent to the *hpt* gene and the *trnA* operon in the Gram-positive bacterium *Lactococcus lactis*. The deduced amino acid sequence of the gene product showed full-length similarity to FtsH of *E. coli*, Yme1p of *Saccharomyces cerevisiae* and a conserved region found in a new family of putative ATPases. In-frame fusions of *L. lactis ftsH* and *phoA1* in *E. coli*, and immunodetection of the *L. lactis* FtsH protein in cell fractions using anti-*E. coli* FtsH serum showed that *L. lactis ftsH* was expressed and encodes a membrane protein. When contained on a high copy number plasmid, the *L. lactis ftsH* gene complemented the lethality of a Δ*ftsH3*::*kan* mutation in *E. coli* at 37 °C and below, indicating that the *L. lactis ftsH* gene can functionally replace the *E. coli ftsH* gene to some extent. The resulting *E. coli* strain showed temperature sensitivity and salt sensitivity. A *L. lactis* mutant with an insertion into *ftsH* was salt-, heat- and cold-sensitive. These results suggest that FtsH is somehow involved in stress responses. Southern hybridization analysis indicated that genes homologous to *ftsH* of *L. lactis* were also present in *Bacillus subtilis*, and several *Lactobacillus* and *Leuconostoc* species, suggesting high conservation of *ftsH* in bacterial species.

Keywords: *Lactococcus lactis*, *ftsH*, (*tma*), membrane protein, AAA-protein family

INTRODUCTION

The facultative anaerobic Gram-positive bacterium *Lactococcus lactis* subsp. *lactis* is widely used as an industrial organism for homolactic fermentation. *L. lactis* has become a model organism for fundamental genetic research on plasmids and plasmid-encoded functions involved in the production of fermented food products. However, the genetic characterization of chromosomally encoded functions in *L. lactis* has only recently been started. We initiated a genetic approach to study purine metabolism in *L. lactis* and have characterized the *hpt* gene encoding hypoxanthine guanine phosphoribosyltransferase, an enzyme involved in the salvage of purine bases to the corresponding nucleotides (Nilsson & Lauridsen, 1992). During this work we detected part of an open reading frame adjacent to *hpt*, transcribed in the same direction, encoding a putative membrane protein.

In this report we describe the cloning and characterization of the *L. lactis* gene, previously named *tma*, adjacent to *hpt* and the tRNA operon *trnA* (Nilsson & Johansen, 1994). We show that the gene is homologous to the *Escherichia coli ftsH* gene, and that this gene is conserved in several Gram-positive bacteria, including lactic acid bacteria. We suggest that the gene be designated *ftsH*.

The *ftsH* genes of *E. coli* and *L. lactis* encode members of a new family of ATPases, the AAA-protein family (Kunau *et al.*, 1993). The family includes both eukaryotic and prokaryotic members which have been reported to be involved in diverse cellular activities, e.g. cell cycle control, protein secretion, peroxisome biogenesis and proteolysis.

E. coli ftsH mutants show pleiotropic phenotypes: defects in cell growth and cell viability (Ogura *et al.*, 1991;

The GenBank accession number for the nucleotide sequence reported in this paper is X69123.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant features	Source or reference
Escherichia coli		
XL1-Blue	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*lac*) [F′ *proAB lacI*^{q}ZΔM15 Tn*10*]	Stratagene, La Jolla
DH5α	*ϕ80lacZ*ΔM15 Δ(*lacZYA–argF*) U169 *recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	Hanahan (1983)
SØ609	*ara thi rpsL* Δ(*pro–gpt–lac*) *hpt deoD purD*	Jochimsen *et al.* (1975)
CC118	*araD139* Δ(*ara–leu*)7697 Δ*lac*X74 *phoA*Δ20 *galE galK thi rpsE rpoB argEam recA1*	Manoil & Beckwith (1986)
AR423	*met gal supE hsdR sfiC* Δ(*srl–recA*) *306*::Tn*10* Δ*ftsH3*::*kan* [pAR171 *ftsH rep*^{ts} Cm^{R}]	Akiyama *et al.* (1994a)
AR3120	*met gal supE hsdR sfiC* Δ(*srl–recA*) *306*::Tn*10* Δ*ftsH3*::*kan* [pLN32 *L. lactis ftsH*]	This study
Lactococcus lactis		
CHCC285	Wild-type	Nilsson & Lauridsen (1992)
MG1363	Plasmid-free	Gasson (1983)
DN4302	MG1363 *ftsH*::*pLN43*	This study
Plasmids		
pBluescript KS+	Cloning vector (Ap^{R})	Stratagene
pBluescript SK−	Cloning vector (Ap^{R})	Stratagene
pVS2	Ery^{R} Cm^{R}	von Wright (1987)
pV2	pBluescript KS+ with a 1·3 kb *ClaI–HpaII* fragment (Ery^{R}) from pVS2	Svend E. W. Hansen (personal communication)
pAR171	*ftsH rep*^{ts} Cm^{R}	Akiyama *et al.* (1994a)
pLN2	pBluescript SK− with a 1·9 kb *Eco*RI–*Mbo*I fragment (*ftsH′*) from CHCC285	Nilsson & Lauridsen (1992)
pLN3	pBluescript SK− with a 1·3 kb *Hin*dIII–*Mbo*I fragment (*′ftsH′*) from CHCC285	Nilsson & Lauridsen (1992)
pLN22-27	pLN2 *ftsH*::Tn*5phoA1*	This study
pLN30	pBluescript KS+ with a 7·3 kb *Spe*I fragment (*hpt ftsH*) from CHCC285	This study
pLN32	pBluescript KS+ with a 3·2 kb *Eco*RI fragment (*ftsH*) from pLN30	This study
pLN39	pBluescript KS+ with a 1·0 kb *Hin*dIII–*Eco*RI fragment (*′ftsH*) from pLN32	This study
pLN43	pV2 with a 1·3 kb *Eco*RV fragment (*′ftsH′*) from pLN32	This study

Tomoyasu *et al.*, 1993a), defects in protein assembly into and through the membrane (Akiyama *et al.*, 1994a, b; Tomoyasu *et al.*, 1993a) and a defect in a proteolytic pathway (Herman *et al.*, 1993). A *Bacillus subtilis ftsH* mutant shows salt sensitivity (Geisler & Schumann, 1993). However, it has not satisfactorily been demonstrated how *ftsH* mutations cause these diverse defects, and what the function of FtsH is. In this respect, it is interesting to know the structural similarity and functional relationship of FtsH in Gram-negative and Gram-positive bacteria. Comparative studies on the FtsH proteins in Gram-positive and Gram-negative bacteria will provide better understanding of the function of this highly conserved putative membrane ATPase.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used are listed in Table 1. *L. lactis* was grown in M17 medium (Oxoid), containing 0·5% glucose, routinely at 30 °C. *E. coli* and *B. subtilis* were grown in L-broth (Miller, 1972) at 37 °C unless otherwise stated. The phosphate-buffered minimal medium of Clark & Maaløe (1967) was used for selection of SØ609 Hpt^{+} as described previously (Nilsson & Lauridsen, 1992). For growth of plasmid-containing cells, the

media contained appropriate antibiotics: *E. coli*, ampicillin (50 mg l^{-1}), kanamycin (300 or 30 mg l^{-1}), chloramphenicol (10 mg l^{-1}); *L. lactis*, erythromycin (1 mg l^{-1}). For identification of blue PhoA$^+$ colonies on agar plates, 40 mg XP l^{-1} (5-bromo-4-chloro-3-indolyl phosphate, Sigma) was added.

DNA manipulations and sequencing. *L. lactis* chromosomal DNA was isolated according to Johansen & Kibenich (1992). *E. coli* and *B. subtilis* chromosomal DNA was isolated as described by Silhavy *et al.* (1984) and *E. coli* plasmids as described by Birnboim & Doly (1979). The use of restriction enzymes, T4 DNA ligase and calf intestine alkaline phosphatase were as recommended by the suppliers (Boehringer Mannheim, Promega, Stratagene). Plasmid transformation of *E. coli* and *L. lactis* was performed according to Mandel & Higa (1970) and Holo & Nes (1989), respectively. The nucleotide sequence of both strands of DNA was determined by the dideoxy method (Sanger *et al.*, 1977). Universal primers from Stratagene or customized primers were used as sequencing primers. All nucleotide sequence data were processed and the deduced amino acid sequences compared using the GCG software package, version 7.0 (Devereux *et al.*, 1984) and EMBL nucleotide sequence database release 37.0.

Cloning of *ftsH*. A *L. lactis* strain CHCC285 chromosomal DNA library, containing 1–10 kb *Spe*I restriction fragments in the bacteriophage vector λZap II (Stratagene) was used. By selecting for Hpt$^+$ colonies of *E. coli* strain SØ609 (Nilsson & Lauridsen, 1992), we isolated clone λLN2 containing a 7·3 kb and a 1·3 kb *Spe*I fragment. The 7·3 kb fragment, containing the *hpt* and *ftsH* genes, was subcloned in the plasmid vector pBluescript KS+ (Stratagene) resulting in pLN30 (Table 1).

Construction of pLN2::Tn*5phoA1*. Plasmid pLN2 (Table 1), which contains the first 441 codons of the *ftsH* gene, was used to transform *E. coli* strain CC118. Following infection of CC118(pLN2) with *λrex*::Tn*5phoA1* *b*221 *c*I857 *Pam*3 (Gutierrez *et al.*, 1987), blue colonies on LB XP agar plates (PhoA$^+$) that were Kan^R and Ap^R, were selected and pooled. Plasmids were isolated and used to transform CC118 to PhoA$^+$, Ap^R and Kan^R. Six independent pLN2::Tn*5phoA1* isolates were obtained.

Identification of *L. lactis* FtsH in *E. coli*. Various *E. coli* extracts from strains containing plasmids were used for SDS-PAGE and subsequently blotted semi-dry to nitrocellulose as described by Ipsen & Larsen (1988). The nitrocellulose was subsequently immunostained (Larsen *et al.*, 1992) using the anti-*E. coli* FtsH serum described previously (Tomoyasu *et al.*, 1993b).

Cell-fractionation of *L. lactis*. Cells were grown in 400 ml of M17 medium to an OD_{600} of 0·9–1·0, harvested and resuspended in 5 ml 30 mM sodium phosphate (pH 6·8) containing 0·4 M sucrose and 1 mg lysozyme ml^{-1}. After incubation at 37 °C for 1 h, the cells were harvested, washed twice with 30 mM sodium phosphate containing 0·4 M sucrose, resuspended in 5 ml 10 mM sodium phosphate (pH 6·8) and sonicated. After ultracentrifugation of the cell extract (100 000 *g*, 1 h at 4 °C), the supernatant (cytoplasmic) fraction was collected. The pellet (membrane) fraction was resuspended in 10 mM sodium phosphate containing 1 M NaCl and ultracentrifuged as above. The supernatant and pellet were collected separately. The supernatant was desalted by gel filtration (Sephadex G-25M, Pharmacia).

The collected fractions were used for SDS-PAGE, blotted to nitrocellulose and immunostained with anti-*E. coli* FtsH serum as described above.

Southern hybridization analysis. A 1·0 kb *Hin*dIII–*Kpn*I (the *Kpn*I site originates from the polylinker of pBluescript SK−) restriction enzyme fragment of plasmid pLN3 (Table 1) contains an internal part of the *L. lactis ftsH* coding region. This fragment was used as probe in Southern hybridization analysis with *Sca*I-digested chromosomal DNA from various bacterial strains, using the ECL Gene Detection System, version 2 (Amersham) as follows. In the hybridization buffer NaCl was added to a final concentration of 1·0 M; primary washes were in 2 × SSC, 1 M urea, at 25 °C. All other procedures including probe labelling, DNA transfer and signal detection were as described by Amersham. The molecular mass markers were obtained from Gibco BRL.

To verify the integration of pLN43 (Table 1) into the *ftsH* gene of *L. lactis* strain DN4302 *ftsH*::*pLN43*, a 1·3 kb *Eco*RV internal fragment of *ftsH* was used as probe in Southern hybridization analysis with *Eco*RI-digested chromosomal DNA from DN4302 and *L. lactis* strain MG1363 wild-type. Standard conditions as recommended by Amersham were used.

RESULTS

Nucleotide sequence of *L. lactis ftsH*

Adjacent to the *hpt* gene and *trnA* operon of *L. lactis*, a gene (*ftsH'*) was found (Nilsson & Lauridsen, 1992; Nilsson & Johansen, 1994). The complete *ftsH* gene was cloned and the nucleotide sequence was determined. The sequence of the last 24 codons of *hpt* and the entire *ftsH* gene is shown in Fig. 1. A possible ribosome binding site (Ludwig *et al.*, 1985; Shine & Dalgarno, 1974) and a putative promoter (Koivula *et al.*, 1991) are located upstream of *ftsH* (Fig. 1). The putative promoter contains the sequence 5′ ATATG 3′ in the −16 region (consensus 5′ RTRTG 3′ where R = purine), which is found in strong *B. subtilis* promoters (Henkin *et al.*, 1988; Moran *et al.*, 1982). A potential stem–loop structure followed by five Ts (nucleotides 82–101) was located between the *hpt* and *ftsH* coding regions. The translation stop codon of the *ftsH* gene was located in the loop of a potential transcription terminator structure (nucleotides 2352–2391).

Analysis of the deduced amino acid sequence of the *ftsH* gene revealed a purine nucleotide binding site motif (residues 233–240 and 288–293) (Walker *et al.*, 1982). Two putative transmembrane sequences can be predicted (residues 1–29 and 136–158) (Kyte & Doolittle, 1982). The transmembrane sequence (residues 136–158) and the region containing the purine nucleotide binding site are separated by a very glycine rich sequence (residues 159–170, GGMGARGGGGGG).

Comparison of *L. lactis* FtsH with other proteins

Database searches for genes encoding similar amino acid sequences revealed that the deduced amino acid sequence of *L. lactis ftsH* was 47% identical to that of *E. coli ftsH* (Tomoyasu *et al.*, 1993a) and 36% identical to that of *Saccharomyces cerevisiae Yme1* (Thorsness *et al.*, 1993). The deduced *L. lactis* FtsH amino acid sequence contains a region of 200 amino acid residues that reveals similarity to conserved domains in the AAA-protein family of putative ATPases (Erdmann *et al.*, 1991; Kunau *et al.*, 1993). In this region the *L. lactis* FtsH amino acid

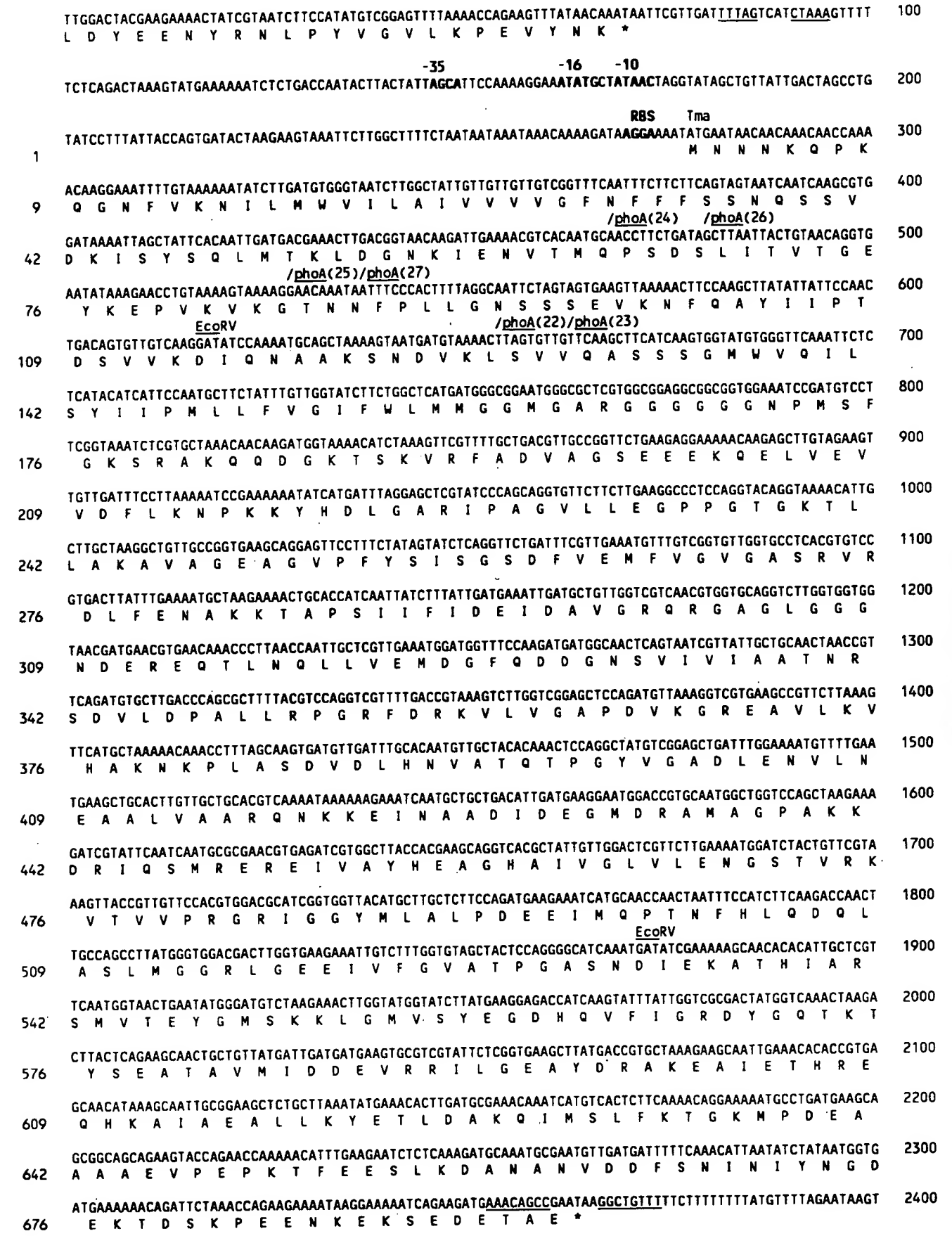

Fig. 1. For legend see facing page.

sequence is 71% identical to that of *E. coli* FtsH and 31 amino acid residues are identical among all sequences shown in Fig. 2.

Localization of *L. lactis* FtsH in the membrane

A system using Tn*5phoA1* to generate protein fusions between the product of a target gene and alkaline phosphatase (AP) has been developed to detect genes encoding membrane and periplasmic proteins in *E. coli* (Manoil & Beckwith, 1985, 1986). Fusion of AP to a periplasmic protein or to a periplasmic domain of a membrane protein is essential for AP activity. Selection for Tn*5phoA1* insertions in pLN2 (Table 1, contains the first 441 codons of *L. lactis ftsH*) resulted in six different plasmids (pLN22–pLN27, Table 1) expressing AP activity in *E. coli*. The fusion points between Tn*5phoA1* and pLN2 in pLN22–27 were determined by DNA sequencing. All the insertions were located in the *ftsH* coding region between nucleotide 467 and 663 (codons 64–129) (Fig. 1).

To further analyse the localization of the *L. lactis* FtsH protein in the cell, we carried out Western blotting analysis of fractionated *L. lactis* cell extracts with anti-*E. coli* FtsH serum, which also reacts with FtsH of *L. lactis*. Sonicated cell extracts from *L. lactis* strain MG1363 and DN4302 *ftsH*::*pLN43* (described below) were fractionated into cytoplasmic and membrane fractions by ultracentrifugation. The membrane fraction was washed with 1 M NaCl and separated into supernatant and pellet. In Fig. 3(a) and (b) (lanes 1–3) a SDS-polyacrylamide gel of the various fractions of cell extract of MG1363 and DN4302 blotted to nitrocellulose and gold-stained is shown. No significant differences in the protein patterns of the cytoplasmic or membrane fractions of MG1363 and DN4302 were found (Fig. 3a and b, lanes 1 and 3), whereas, the supernatants of the salt-washed membranes differed in protein patterns (Fig. 3a and b, lanes 2). The anti-*E. coli* FtsH serum only detected FtsH from MG1363 and a truncated form of FtsH from DN4302 in the pellets of the salt-washed membrane fractions (Fig. 3a and b, lanes 6).

It is concluded from the results described above, that FtsH of *L. lactis* is an integral membrane protein spanning the membrane twice with the region flanked by these hydrophobic stretches protruding outside the cell, and that it has a large cytoplasmic carboxy-terminal part with a putative ATP-binding domain (Fig. 4). Such overall features of the *L. lactis* FtsH topology are consistent with those of the *E. coli* homologue, FtsH (Tomoyasu *et al.*, 1993b), except that *L. lactis* FtsH has a larger outside domain than *E. coli* FtsH.

Complementation of Δ*ftsH3*::*kan* in *E. coli* with *ftsH* of *L. lactis*

Since the deduced amino acid sequences of *ftsH* from *L. lactis* and *E. coli* showed a high degree of identity, we were interested to know whether or not the *L. lactis ftsH* gene could complement a *ftsH* mutation in *E. coli*.

The *E. coli* strain AR423 Δ*ftsH3*::*kan* (pAR171 *ftsH rep*^{ts} Cm^{R}) shows temperature-sensitive growth, because the replication of the plasmid pAR171, containing the essential *ftsH* gene and a chloramphenicol (Cm) resistance marker, is defective at 42 °C (Akiyama *et al.*, 1994a).

The plasmid pLN32 contains the entire *L. lactis ftsH* gene, whereas the plasmids pLN2 and pLN39 contain various parts of the *L. lactis ftsH* gene, all cloned in the vector pBluescript KS+ or pBluescript SK− containing ampicillin resistance (Ap^{R}) markers (Fig. 5a, Table 1). These plasmids were used to transform strain AR423 by selection for Ap^{R} at 30 °C. The strains obtained were incubated in LB at 42 °C for 6 h, and then plated on LB agar at 30 °C. The plasmid content of the colonies was tested by streaking on LB agar containing Ap or Cm; and the possession of the Δ*ftsH3*::*kan* mutation was tested by streaking on LB agar containing Kan. From transformants containing the entire *L. lactis ftsH* gene on pLN32, Cm^{S} colonies could be isolated with a frequency of approximately 40–50%, indicating that these had lost pAR171, containing the *E. coli ftsH* wild-type. Such colonies were all Ap^{R} and Kan^{R}, indicating that these had retained pLN32, containing the *L. lactis ftsH* gene, and the mutation Δ*ftsH3*::*kan*. Colonies of transformants with pLN2 and pLN39, containing only part of *L. lactis ftsH*, were all Ap^{R}, Cm^{R} and Kan^{R}, showing that no loss of pAR171 was obtained. Thus pAR171, and hereby the *E. coli* wild-type *ftsH* gene, can be lost only from strains containing the complete *L. lactis ftsH* gene. One strain, AR3120 Δ*ftsH3*::*kan* (pLN32 *L. lactis ftsH*), was saved for further studies.

Western blots with anti-*E. coli* FtsH serum were performed using extracts of AR423, AR423 containing the plasmids in Fig. 5(a) and AR3120 to detect the gene products of *E. coli ftsH* and/or *L. lactis ftsH* (Fig. 5b). The *E. coli* FtsH protein could be detected in the AR423 derivatives but not in AR3120, whereas the *L. lactis* FtsH protein was detected in AR423(pLN32) and AR3120. No *L. lactis ftsH* product was detected in AR423, AR423(pLN39), AR423(pLN2) or AR423(pKS+). This

Fig. 1. Nucleotide sequence of the *L. lactis ftsH* gene. The numbers to the right indicate nucleotide positions (*'hpt*, nucleotides 1–72; *ftsH*, nucleotides 278–2365, 696 codons). The deduced amino acid sequences of *'hpt* and *ftsH* (FtsH) are shown in one-letter code below, with the translation stop codons indicated by asterisks. The numbers to the left indicate the deduced amino acid residue position of FtsH. The putative ribosome binding sites (RBS) and promoter regions (−35, −16, −10) are indicated. Two putative stem–loop structures are underlined. The Tn*5phoA1* insertions in pLN22–27 are indicated above the sequence. The *Eco*RV sites used to construct the integration plasmid pLN43 are shown above the sequence.

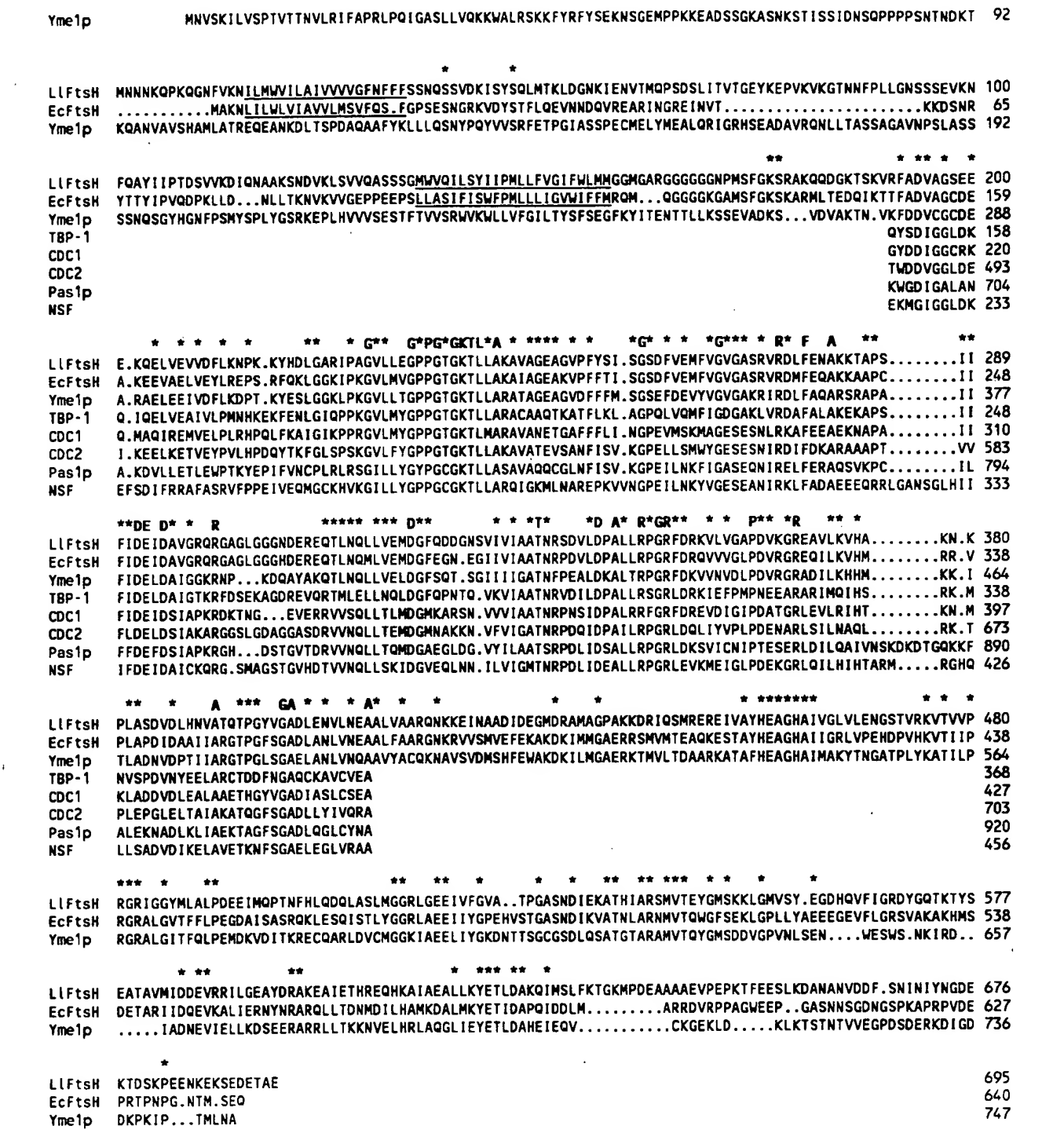

Fig. 2. Amino acid sequence alignment of *L. lactis* (Ll) FtsH, *E. coli* (Ec) FtsH, Yme1p and the conserved domains found in *L. lactis* FtsH (residues 191–410); TBP-1 (Nelbock *et al.*, 1990); CDC48p (CDC1, CDC2; CDC48p contains two conserved domains) (Fröhlich *et al.*, 1991); Pas1p (Erdmann *et al.*, 1991); and NSF (Wilson *et al.*, 1989). S4 of the 26S protease, MSS1 and SUG1 resembling TBP-1 (Dubiel *et al.*, 1992; Shibuya *et al.*, 1992; Swaffield *et al.*, 1992), VCP and p97 resembling CDC48p (Koller & Brownstein, 1987; Peters *et al.*, 1990), and Sec18p resembling NSF (Eakle *et al.*, 1988) also contain the conserved domain (not shown). Identical amino acid residues found in these sequences are indicated in boldface type above the sequences. Identical amino acid residues of *L. lactis* FtsH, *E. coli* FtsH and Yme1p are indicated by asterisks. The two putative membrane-spanning sequences of *L. lactis* FtsH and *E. coli* FtsH are underlined.

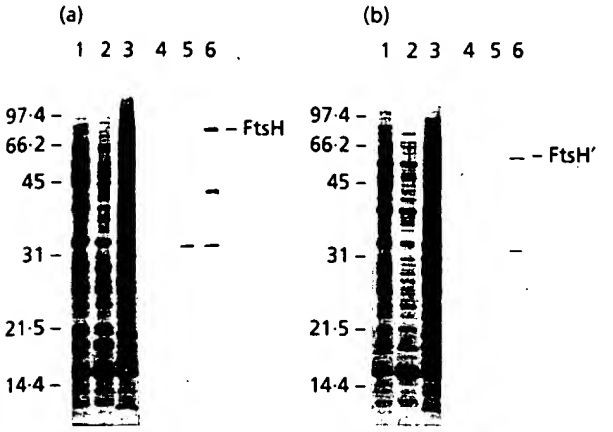

Fig. 3. Localization of *L. lactis* FtsH in cell fractions. Fractionation of extracts from MG1363 and DN4302, and treatment of membrane fractions with 1 M NaCl were done as described in Methods. Electrophoresis of the cell fractions on an SDS-polyacrylamide gel (a and b) was done, blotted to nitrocellulose and gold-stained (lanes 1–3) or subjected to Western blotting with anti-*E. coli* FtsH serum (lanes 4–6). Positions of molecular mass reference markers, FtsH and a truncated form of FtsH (FtsH') are indicated. All other bands are a result of unspecific binding of the secondary anti-serum used (control not shown). (a) Lanes: 1 and 4, cytoplasmic fraction of MG1363; 2 and 5, supernatant derived from membrane fraction of MG1363 washed with 1 M NaCl; 3 and 6, pellet derived from membrane fraction of MG1363 washed with 1 M NaCl. (b) Lanes: same as (a) except extract from DN4302 was used.

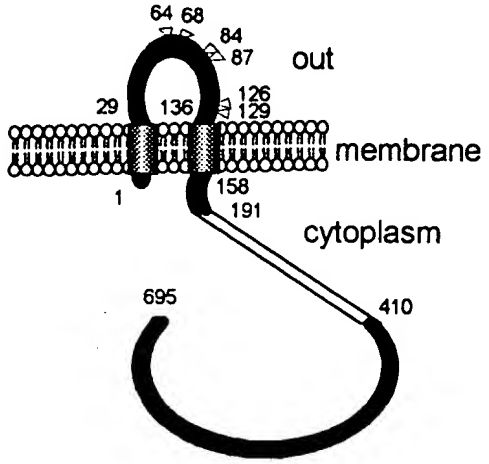

Fig. 4. Deduced topology of the *L. lactis* FtsH protein. Numbers indicate amino acid residue positions. Shaded boxes illustrate putative membrane-spanning regions. The encoded FtsH–PhoA fusions of pLN22–27 that results in AP activity are fused at positions indicated by triangles. The region with similarity to the conserved region of the AAA-protein family is indicated by an open box. This region contains the ATP binding motifs.

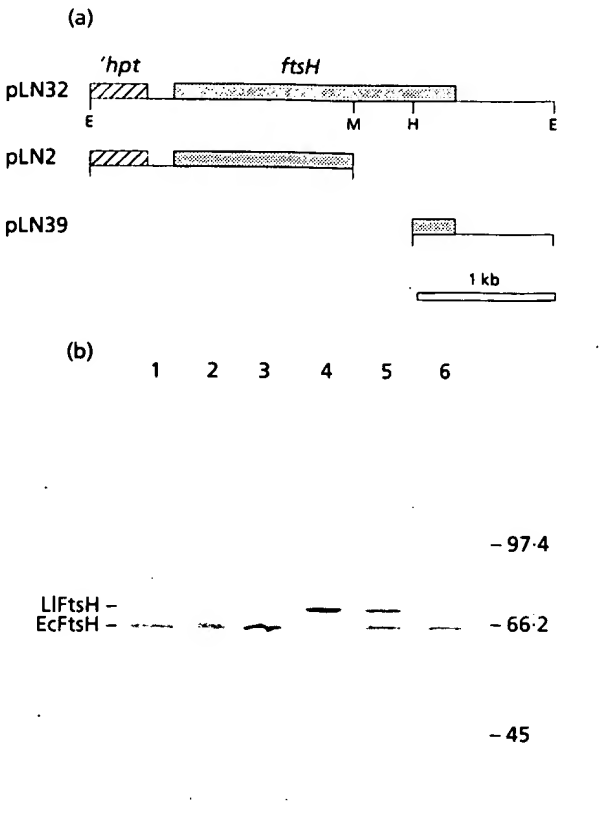

Fig. 5. Immunological detection of *L. lactis* FtsH and *E. coli* FtsH. (a) The lines represent *L. lactis* chromosomal DNA fragments cloned in pBluescript KS+ or SK−. The restriction enzyme sites shown are: E, *Eco*RI; H, *Hin*dIII; M, *Mbo*I. The location of the *L. lactis 'hpt* and *ftsH* genes is indicated by hatched and shaded areas, respectively. (b) Western blot. The FtsH proteins of *L. lactis* (Ll) and *E. coli* (Ec) were detected using anti-*E. coli* FtsH serum. The positions of *L. lactis* FtsH and *E. coli* FtsH are indicated to the left. Lanes: 1, AR423(pKS+); 2, AR423(pLN39); 3, AR423(pLN2); 4, AR3120; 5, AR423(pLN32); 6, AR423. The molecular mass reference markers are indicated on the right.

demonstrates that AR3120 was able to grow without the essential *E. coli ftsH* gene product when containing the *L. lactis ftsH* gene product. The plasmid pLN2 encodes a truncated *L. lactis* FtsH protein containing the epitope for the anti-*E. coli* FtsH serum, when transcribed and translated *in vitro* (Nilsson & Lauridsen, 1992; data not shown). This truncated FtsH was not detected by the anti-*E. coli* FtsH serum in the Western blot, possibly due to rapid degradation *in vivo*.

E. coli AR3120 showed temperature-sensitive growth in that it could grow at 30 °C and 37 °C but not at 42 °C. A *B. subtilis* mutant with a salt-sensitive phenotype has been shown to be impaired in a putative homologue of *ftsH* (Geisler & Schumann, 1993). AR423, AR423(pLN32) and AR3120 were tested for growth on LB agar plates containing 4% (w/v) NaCl. AR423 and AR423(pLN32), but not AR3120, were able to grow on this medium.

Construction of a *L. lactis ftsH* mutant

A 1·3 kb *Eco*RV fragment of pLN32, containing an internal part of *L. lactis ftsH* (Fig. 1), was cloned into the *L. lactis* integration vector pV2 giving pLN43 (Table 1).

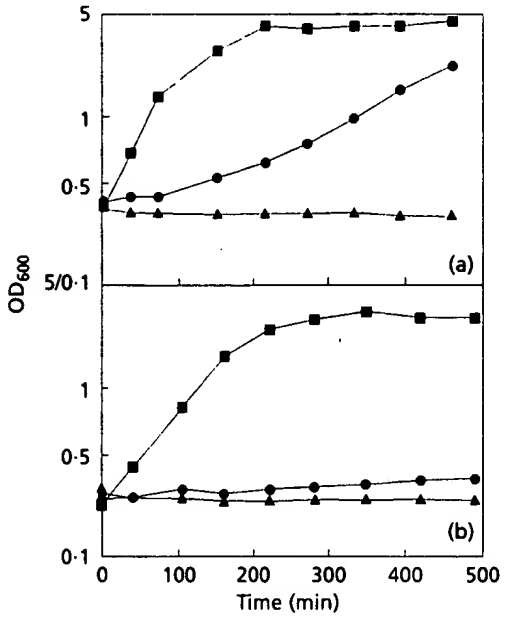

Fig. 6. Growth of DN4302 and MG1363 at various NaCl concentrations. MG1363 (a) and DN4302 (b) were grown exponentially in M17 medium and then diluted twofold in M17 medium containing 0% (■), 4% (●) or 7% (▲) (w/v) NaCl (final concentrations).

Transformation of pLN43 into *L. lactis* strain MG1363, selecting for erythromycin resistance, resulted in strain DN4302 *ftsH*::*pLN43*. The integration of pLN43 into *ftsH* of DN4302 was verified by Southern hybridization analysis (not shown). The construction of DN4302 indicates that the *ftsH* gene is not essential in *L. lactis* or that the truncated form of FtsH encoded by the disrupted *ftsH* gene (Fig. 3b, lane 6) is enough to retain any essential function. DN4302 and MG1363 were tested for growth on M17-agar plates containing 4% or 1% NaCl. MG1363 could grow at both 4% and 1% NaCl, whereas DN4302 grew slowly at 1% and not at all at 4% NaCl. Fig. 6 shows the growth of DN4302 and MG1363 at various salt concentrations in M17 broth. MG1363 grew at 4% NaCl after a lag phase of about 2 h, whereas DN4302 at 4% NaCl showed almost no growth. No growth of either strains occurred with 7% NaCl. DN4302 and MG1363 were also tested for growth on M17-agar plates containing 0·5 M sucrose. Both strains appeared to grow equally well on this medium.

Growth at 38 °C, 30 °C and 16 °C on M17-agar plates was also tested. DN4302 only grew at 30 °C, whereas MG1363 grew at all temperatures. However, incubating DN4302 anaerobically restored growth at 38 °C.

Homologous genes in other bacteria

A 1·0 kb *Hin*dIII–*Kpn*I fragment (nucleotides 667–1600, Fig. 1) was used as probe in Southern hybridization analysis with *Sca*I-digested chromosomal DNA from various bacterial strains (Fig. 7). Single chromosomal bands can be detected from the Gram-positive bacteria *L. lactis*, *B. subtilis*, *Leuconostoc* sp. and *Lactobacillus* sp. (Fig. 7, lanes 1, 3–8). With *E. coli* DNA only a very faint band was detected (Fig. 7, lane 2). Under the same experimental conditions, homology to *hpt*, using a *hpt* probe, was only detected in *L. lactis* (results not shown).

DISCUSSION

The *ftsH* gene of *L. lactis* was cloned and shown to encode a transmembrane protein with putative ATPase activity. The deduced amino acid sequence indicates that FtsH belongs to the AAA-protein family of putative ATPases containing several members of eukaryotic proteins (Erdmann *et al.*, 1991; Kunau *et al.*, 1993) and also includes the membrane protein FtsH of *E. coli* (Tomoyasu *et al.*, 1993a, b). FtsH from *L. lactis* and *E. coli* seem to be homologous proteins based on the high similarity of the amino acid sequences and that the *L. lactis ftsH* gene can complement certain defects of an *E. coli* Δ*ftsH3*::*kan* strain. Southern hybridization analysis of various Gram-positive bacteria using *L. lactis ftsH* as probe suggests that homologous genes exist. In *B. subtilis* a putative *ftsH* gene has been located on the chromosome of *B. subtilis* adjacent to the *hpt* gene (Geisler & Schumann, 1993; Ogasawara *et al.*, 1994), indicating a similar arrangement of *hpt* and *ftsH* as in *L. lactis*. It seems from these results that *ftsH* genes are widely conserved in bacteria.

The function of FtsH is unknown. Our *L. lactis ftsH* mutant and the *B. subtilis ftsH* mutant (Geisler & Schumann, 1993) were constructed by a Campbell-type integration with an internal gene fragment. This should inactivate the genes. In *E. coli*, *ftsH* is essential for growth. Since the resulting mutants are viable, it is possible that the *ftsH* genes are not essential in *L. lactis* and *B. subtilis*. However, in at least *L. lactis*, the truncated form of FtsH produced by the mutant DN4302 may still retain essential functions.

The *L. lactis ftsH* mutant DN4302 is impaired in salt tolerance and other stress responses, and shows a different pattern of membrane-associated proteins compared to the wild-type strain (Fig. 3, lanes 2). These phenotypes could be explained by an improper assembly of membrane proteins, some necessary for the salt tolerance, caused by the *ftsH* mutations in *L. lactis*. In *E. coli* the maturation of penicillin binding protein 3 (PBP3) and β-lactamase is dependent on FtsH function, in that post-translational processing at the C-terminal part of PBP3 seems to be defective and accumulation of the plasmid-encoded precursor of β-lactamase in the cytoplasm was observed in the thermosensitive *ftsH1* mutant of *E. coli* (Begg *et al.*, 1992; Tomoyasu *et al.*, 1993a). Analysis of several newly constructed *E. coli ftsH* mutants encoding FtsH variants, including C-terminally truncated forms with dominant phenotypes, suggests that FtsH is involved in assembly/folding of proteins into and through the membrane and that FtsH is needed to assure efficient stop-transfer of some membrane proteins (Akiyama *et al.*, 1994a, b).

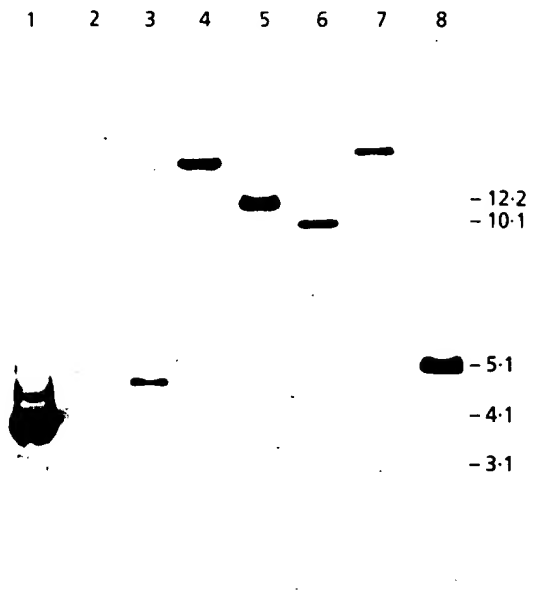

Fig. 7. Southern hybridization of an internal part of the *L. lactis ftsH* gene to chromosomal DNA from various bacteria. Lanes: 1, *L. lactis* CHCC285; 2, *E. coli* XL-1 Blue; 3, *B. subtilis* 168; 4, *Leuconostoc mesenteroides* DB1165; 5, *Leu. oenos* LOP89005; 6, *Lactobacillus plantarum* LP2T1G; 7, *Lactob. acidophilus* LA2753; 8, *Lactob. lactis* CH-2. Molecular mass markers are shown on the right in kb.

However, how FtsH is functionally related to these phenotypes is not known. Recently it was shown that a *hflB* mutation in *E. coli* causing increased lysogenization frequencies of bacteriophage λ through a reduced degradation of the *c*II protein, is located in *ftsH*, and the HflB/FtsH protein has been suggested to be an energy-dependent chaperone and/or protease (Herman *et al.*, 1993). Other members of the AAA-protein family are also involved in proteolysis, e.g. subunit 4 of the human ATP-dependent 26S protease, and it was proposed that other eukaryotic members such as Tbp1, Sug1 and MSS1 are also subunits of the 26S protease (Dubiel *et al.*, 1992). Taken together FtsH might function as a chaperone and/or a protease although no direct biochemical evidence for these functions has been reported so far. Comparative biochemical and genetic analysis of FtsH from *L. lactis* and *E. coli* will provide better understanding of the biological significance of FtsH, if it is a chaperone or a protease, and what are its substrates.

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Saturation Site-directed Mutagenesis of Thymidylate Synthase*

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We have subjected 12 different codons of a synthetic *Lactobacillus casei* thymidylate synthase (TS) gene to saturation site-directed mutagenesis to create amino acid "replacement sets" at each of those positions. The target residues were chosen because they are highly conserved and because they are important for the structure and function of the protein as indicated by solution and structural studies. The mutagenesis procedure involved excision of a fragment of the synthetic gene containing the target codon, followed by its replacement with a mixture of oligonucleotides which code for all 20 amino acids and the amber stop codon. TS mutants were identified by DNA sequencing, and catalytically active mutants were identified by genetic complementation using a Thy⁻ strain of *Escherichia coli*. Only 3 of the 12 target amino acids examined were essential for TS activity; and of the 125 total mutants identified, 57 were catalytically active. These results point to a high degree of plasticity of TS in accommodating function with structural change.

Thymidylate synthase (TS)[1] catalyzes the conversion of 2′-deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate to 2′-deoxythymidylate (dTMP) and dihydrofolate. TS is the sole *de novo* source of dTMP, and there has been much interest in studies of its structure, function, and inhibition. In part, the interest in TS results from the large amount of information on this enzyme which now exists. The enzymology of TS has been extensively studied, and details about its mechanism and inhibition are well understood (1). Also, the primary sequences of TS from some 16 sources have been determined and have revealed that it is the one of most conserved proteins known (2); the similarity among TS sequences implies a functional importance for the conserved residues. Finally, the x-ray structures of the *Lactobacillus casei* TS·P_i complex and the *Escherichia coli* TS·dUMP·10-propargyl-5,8-dideazafolate (CB3717) ternary complex have provided insight about the possible roles of specific residues of the protein (3-5).

Thus far, mutations of key residues of TS have provided unexpected results. His^{199}, a putative general base catalyst (6, 7), and Arg^{179}, a substrate-binding residue (8), could each be substituted by several different residues without large effects on binding or catalysis; even the Cys involved in nucleophilic catalysis could be modified to Ser and still retain activity, albeit severely diminished (9). From such observations, we became cautious about directing our efforts toward the study of one or a few mutants. Instead, we adopted a plan to produce all possible mutants at a residue of interest and developed screening methods for various properties to reveal mutants of interest.

In this report, we describe the strategy and preliminary results of an approach to understanding the structure of TS by saturation site-directed mutagenesis. The mutations were constructed by cassette mutagenesis using an *E. coli* expression vector that carries a synthetic *L. casei* TS gene with over 30 unique restriction sites (7). The mutagenic DNA cassettes contained mixtures of 32 codons that encode 20 amino acids and the amber (TAG) stop codon at the target site (10-12). "Replacements sets" were constructed in which each of 12 target amino acids were replaced by a large number of substitutions, and a complementation assay was used to rapidly screen for mutants that were catalytically active and thus of more interest for further study.[2]

MATERIALS AND METHODS

Restriction endonucleases and T4 ligase were purchased from New England BioLabs and Bethesda Research Laboratories and were used as recommended. The Sequenase DNA sequencing kit was purchased from U. S. Biochemical Corp. The *E. coli* strains used were TB-1 (ϕ80lacZΔM15, ara, Δ(lac-proAB), rpsL, hsdR) from T. O. Baldwin (Texas A & M, College Station, TX) and Thy⁻ χ2913 (ΔthyA572) from R. Thompson (University of Glasgow, United Kingdom). Oligonucleotides were synthesized at the University of California-San Francisco Biomolecular Resource Center using an Applied Biosystems 380B DNA synthesizer; tritylated oligonucleotide mixtures were purified and de-tritylated on Du Pont-New England Nuclear NENSORB Prep cartridges using the recommendations of the manufacturer. General methods for plasmid purification, subcloning, and bacterial transformation were as described (13).

In vitro cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9 (7). In some cases, pSCTS9 was first modified by substituting a 19-base noncoding sequence containing unique *NotI* and *SphI* sites for the fragment containing the target codon; this eliminates wild-type sequences and allows for selective destruction of the parent plasmid and resultant increase in mutagenesis efficiency (14). Methods for the construction and characterization of the TS mutants were as described (7). Short fragments of the TS gene containing the target codon were replaced with a synthetic DNA duplex presenting a mixture of all four bases at the first and second positions of the target codon and a mixture of G and C at the third position. The plasmids from the ligation mixture were used to trans-

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[1] The abbreviation used is: TS, thymidylate synthase.

[2] The use of a series of amber tRNA suppressors and genetic complementation to construct and characterize a large number of mutants of *E. coli* TS was reported after this manuscript was submitted (16).

form *E. coli* TB-1, and plasmid pools were transformed into *E. coli* Thy⁻ χ2913. Individual plasmids were twice re-transformed into χ2913 and sequenced. Catalytically active substitutions were identified by screening for the ability of Thy⁻ χ2913 cells harboring the mutant plasmids to grow on minimal agar lacking Thd and were again characterized by DNA sequencing.

RESULTS AND DISCUSSION

The amino acids targeted for mutagenesis were chosen because they are highly conserved and because they have putative roles in substrate or cofactor binding or in catalysis, as determined by solution or structural studies (Fig. 1). The strategy used in the construction and characterization of the replacement sets is outlined in Fig. 2. Following ligation of oligonucleotide mixtures and initial transformation, colonies were pooled, and the mutagenized plasmid DNA pool was recovered and used to transform *E. coli* Thy⁻ χ2913 to ampicillin resistance. Individual mutants were identified by DNA sequencing, and the resulting plasmids were used to re-transform χ2913. Plasmid DNA from the secondary χ2913 transformants was again sequenced and tested for TS activity as described below. Passage of the mutagenized DNA mixtures through several rounds of transformation ensured segregation and/or repair of the heteroduplex DNA molecules that were created during the construction of the mutants. It was not practical to identify all possible mutants in a replacement set by this strategy, which would require sequencing of over 160 clones to obtain a 95% confidence level of obtaining a complete set. Generally, 20–30 isolates of a replacement set were sequenced to give 10–15 different mutants of a given residue.

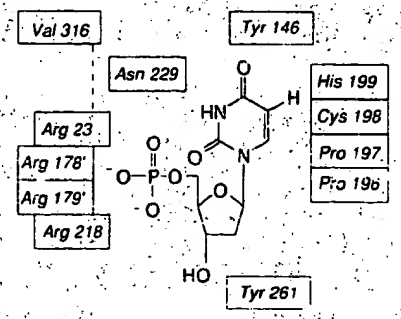

FIG. 1. **Amino acid residues of TS targeted for mutagenesis depicted near the sites of interaction with dUMP.** - - -, hydrogen bond between Val[316] and Arg[23].

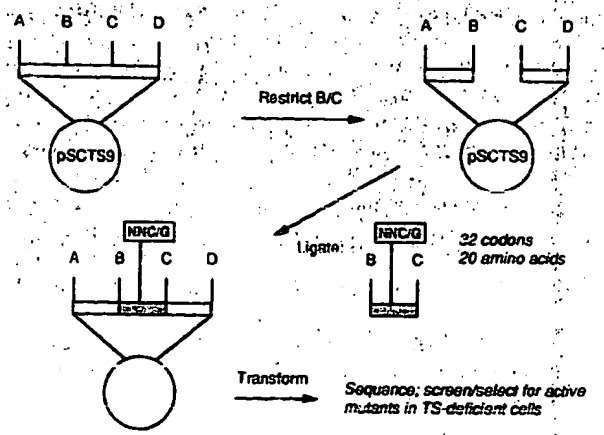

FIG. 2. **Strategy for saturation site-directed mutagenesis of synthetic TS gene and identification of catalytically active mutants.** A fragment of the TS gene between any two restriction sites is removed and replaced by a mixture of oligonucleotides containing NNC/G at the target codon. The resultant plasmids are sequenced and screened in *E. coli* Thy⁻ cells for catalytic activity.

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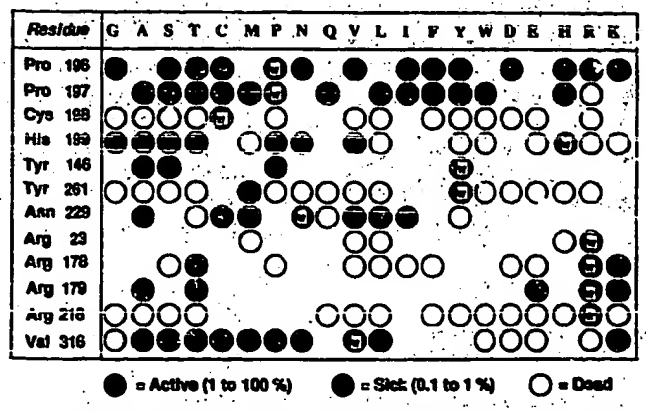

FIG. 3. **Results of complementation screen of mutants in TS-deficient cells.** ●, full growth of colonies harboring plasmids; ○, poor growth; ○, no growth. The wild-type mutants isolated are indicated by *w* within the circles. The Arg[178] mutants were derived from single-strand mutagenesis experiments (8).

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Conserved Arg residues at positions 23, 178, 179, and 218 form a positively charged binding surface for the 5′-phosphate of dUMP (3, 4). Five of the guanidine NH groups are within appropriate distance (2.5–3.2 Å) to provide hydrogen bonding or electrostatic stabilization of the phosphate anion of dUMP (4). Recently, we reported (8) that Arg^{179} can be replaced by amino acids as diverse as Ala and Glu without dramatic effects on binding or catalysis. For Arg^{178}, after sequencing 30 clones, we isolated 10 mutants, the amber codon, and three synonymous Arg codons. Two mutants were fully active in the complementation assay. After sequencing 11 clones, we obtained four mutants, the amber codon, and two synonymous Arg codons at position 23; none of the mutants were active by the complementation assay. For Arg^{218}, we isolated 14 amino acid mutations and the parental Arg codon at position 218 after sequencing 30 clones; sequencing an additional 43 clones provided no additional mutants. None of the mutants were active in the complementation assay. Thus, of the 4 Arg residues involved in phosphate binding, only Arg^{23} and Arg^{218} have thus far been shown to be essential.

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Tyr^{261} is a completely conserved residue which forms a hydrogen bond to the 3′-hydroxyl of dUMP in the ternary complex. We isolated 14 amino acid mutations, the wild-type Tyr, and the amber codon at position 261 after sequencing 41 clones. Of these, only the Met mutant was active by complementation.

Asn^{229} is completely conserved and lies in the highly conserved, hydrophobic J-helix which forms the core of TS. In the ternary *E. coli* TS·dUMP·CB3717 complex, Asn^{229} interacts with bound dUMP via hydrogen bonds between the γ-oxygen of Asn and NH-3 of dUMP and between γ-NH_2 and O-4. We isolated 9 of the 20 possible amino acid substitutions, the parental Asn, and the amber codon after sequencing 13 clones. Five of these were active by complementation.

The carboxyl terminus of most TS sequences is Val, although Ile, Leu, and Ala have been found. It appears that the carboxyl-terminal residue is important to TS since removal of Val^{316} in *L. casei* TS by carboxypeptidase leads to a loss of folate cofactor binding and catalytic activity (15). Interestingly, on forming TS·dUMP·CB3717, the carboxyl-terminal residues undergo a large movement toward the active site where they approach the folate analog (4). The terminal Val^{316} carboxylate is hydrogen-bonded to the essential Arg^{23} and conserved Trp^{85} which probably stabilize the conformer. We isolated 14 amino acid mutations at position 316, the wild-type Val, and the amber codon after sequencing 29 clones. Of these, nine were active by complementation. As expected, the amber mutant lacking terminal residue 316 was not active by the complementation assay.

SUMMARY

We have described a simple strategy for saturation mutagenesis of any residue of TS and for the rapid identification of mutants which are catalytically active. The targeted codon of a synthetic gene is replaced by a mixture of duplex oligonucleotides which encodes all 20 amino acids. After isolation of individual mutants, a complementation assay using TS-deficient *E. coli* rapidly identifies those mutants with catalytic activity.

The important components of the approach we have used which make it generic include the following. (*a*) A synthetic gene with conveniently placed, unique restriction sites permits mutagenesis at any position at nearly 100% mutagenesis efficiency. (*b*) A mutagenesis vector which also serves as a high expression vector avoids further subcloning. (*c*) A selection system for the desired property permits rapid identification of mutants of interest.

We have made replacement sets of amino acid substitutions at 12 conserved residues that are believed to play roles in structure and function of TS. We isolated some 125 different site-directed mutants, 57 (46%) of which were catalytically active. With the exception of Cys^{198}, Arg^{23}, and Arg^{218}, none of the residues that were mutagenized are strictly essential for activity. All of the active replacement positions had mutants with at least 1% of the activity of wild-type TS, and many have been found with k_{cat} values approaching that of the natural enzyme.[3] Despite the wealth of information available on TS, it would not have been possible to predict the

[3] S. Climie, L. Ruiz-Perez, D. Gonzalez-Pacanowska, P. Prapunwattana, S.-W. Cho, R. Stroud, and D. V. Santi, unpublished data.

range of allowable substitutions, and the results stand as a caution to assigning structure-function roles of a given residue based on one or a few mutants. To explain how TS can tolerate modifications at so many conserved residues of presumed importance, we suggest that there is a high plasticity of the structure of this protein in accommodating function; that is, when a mutation is made that is potentially detrimental to function, the structure may be sufficiently flexible to accommodate the change. Furthermore, it appears that many of the amino acids studied may not simply serve a role in catalysis, or else they would not have survived evolutionary pressure. We are searching for other contributing factors to the conservation of these residues.

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Saturation Site-directed Mutagenesis of Thymidylate Synthase*

(Received for publication, May 18, 1990)

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From the ‡Departments of Biochemistry and Biophysics and of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0448 and the §Institute Lopez-Neyra of Parasitology, Consejo Superior de Investigaciones Cientificas, Granada, 18001 Spain

We have subjected 12 different codons of a synthetic *Lactobacillus casei* thymidylate synthase (TS) gene to saturation site-directed mutagenesis to create amino acid "replacement sets" at each of those positions. The target residues were chosen because they are highly conserved and because they are important for the structure and function of the protein as indicated by solution and structural studies. The mutagenesis procedure involved excision of a fragment of the synthetic gene containing the target codon, followed by its replacement with a mixture of oligonucleotides which code for all 20 amino acids and the amber stop codon. TS mutants were identified by DNA sequencing, and catalytically active mutants were identified by genetic complementation using a Thy⁻ strain of *Escherichia coli*. Only 3 of the 12 target amino acids examined were essential for TS activity; and of the 125 total mutants identified, 57 were catalytically active. These results point to a high degree of plasticity of TS in accommodating function with structural change.

Thymidylate synthase (TS)[1] catalyzes the conversion of 2′-deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate to 2′-deoxythymidylate (dTMP) and dihydrofolate. TS is the sole *de novo* source of dTMP, and there has been much interest in studies of its structure, function, and inhibition. In part, the interest in TS results from the large amount of information on this enzyme which now exists. The enzymology of TS has been extensively studied, and details about its mechanism and inhibition are well understood (1). Also, the primary sequences of TS from some 16 sources have been determined and have revealed that it is the one of most conserved proteins known (2); the similarity among TS sequences implies a functional importance for the conserved residues. Finally, the x-ray structures of the *Lactobacillus casei* TS·P_i complex and the *Escherichia coli* TS·dUMP·10-propargyl-5,8-dideazafolate (CB3717) ternary complex have provided insight about the possible roles of specific residues of the protein (3–5).

Thus far, mutations of key residues of TS have provided unexpected results. His^{109}, a putative general base catalyst (6, 7), and Arg^{179}, a substrate-binding residue (8), could each be substituted by several different residues without large effects on binding or catalysis; even the Cys involved in nucleophilic catalysis could be modified to Ser and still retain activity, albeit severely diminished (9). From such observations, we became cautious about directing our efforts toward the study of one or a few mutants. Instead, we adopted a plan to produce all possible mutants at a residue of interest and developed screening methods for various properties to reveal mutants of interest.

In this report, we describe the strategy and preliminary results of an approach to understanding the structure of TS by saturation site-directed mutagenesis. The mutations were constructed by cassette mutagenesis using an *E. coli* expression vector that carries a synthetic *L. casei* TS gene with over 30 unique restriction sites (7). The mutagenic DNA cassettes contained mixtures of 32 codons that encode 20 amino acids and the amber (TAG) stop codon at the target site (10–12). "Replacements sets" were constructed in which each of 12 target amino acids were replaced by a large number of substitutions, and a complementation assay was used to rapidly screen for mutants that were catalytically active and thus of more interest for further study.[2]

MATERIALS AND METHODS

Restriction endonucleases and T4 ligase were purchased from New England BioLabs and Bethesda Research Laboratories and were used as recommended. The Sequenase DNA sequencing kit was purchased from U. S. Biochemical Corp. The *E. coli* strains used were TB-1 (ϕ80lacZΔM15; ara, Δ(lac-proAB), rpsL, hsdR) from T. O. Baldwin (Texas A & M, College Station, TX) and Thy⁻ χ2913 (ΔthyA572) from R. Thompson (University of Glasgow, United Kingdom). Oligonucleotides were synthesized at the University of California-San Francisco Biomolecular Resource Center using an Applied Biosystems 380B DNA synthesizer; tritylated oligonucleotide mixtures were purified and de-tritylated on Du Pont-New England Nuclear NENSORB Prep cartridges using the recommendations of the manufacturer. General methods for plasmid purification, subcloning, and bacterial transformation were as described (13).

In vitro cassette mutagenesis was performed using a synthetic TS gene carried on the plasmid pSCTS9 (7). In some cases, pSCTS9 was first modified by substituting a 19-base noncoding sequence containing unique *NotI* and *SphI* sites for the fragment containing the target codon; this eliminates wild-type sequences and allows for selective destruction of the parent plasmid and resultant increase in mutagenesis efficiency (14). Methods for the construction and characterization of the TS mutants were as described (7). Short fragments of the TS gene containing the target codon were replaced with a synthetic DNA duplex presenting a mixture of all four bases at the first and second positions of the target codon and a mixture of G and C at the third position. The plasmids from the ligation mixture were used to trans-

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¶ Senior Fulbright/Spanish Ministry of Education and Science Scholar.

‖ To whom reprint requests should be addressed.

[1] The abbreviation used is: TS, thymidylate synthase.

[2] The use of a series of amber tRNA suppressors and genetic complementation to construct and characterize a large number of mutants of *E. coli* TS was reported after this manuscript was submitted (16).

form *E. coli* TB-1, and plasmid pools were transformed into *E. coli* Thy⁻ χ2913. Individual plasmids were twice re-transformed into χ2913 and sequenced. Catalytically active substitutions were identified by screening for the ability of Thy⁻ χ2913 cells harboring the mutant plasmids to grow on minimal agar lacking Thd and were again characterized by DNA sequencing.

RESULTS AND DISCUSSION

The amino acids targeted for mutagenesis were chosen because they are highly conserved and because they have putative roles in substrate or cofactor binding or in catalysis, as determined by solution or structural studies (Fig. 1). The strategy used in the construction and characterization of the replacement sets is outlined in Fig. 2. Following ligation of oligonucleotide mixtures and initial transformation, colonies were pooled, and the mutagenized plasmid DNA pool was recovered and used to transform *E. coli* Thy⁻ χ2913 to ampicillin resistance. Individual mutants were identified by DNA sequencing, and the resulting plasmids were used to re-transform χ2913. Plasmid DNA from the secondary χ2913 transformants was again sequenced and tested for TS activity as described below. Passage of the mutagenized DNA mixtures through several rounds of transformation ensured segregation and/or repair of the heteroduplex DNA molecules that were created during the construction of the mutants. It was not practical to identify all possible mutants in a replacement set by this strategy, which would require sequencing of over 160 clones to obtain a 95% confidence level of obtaining a complete set. Generally, 20–30 isolates of a replacement set were sequenced to give 10–15 different mutants of a given residue.

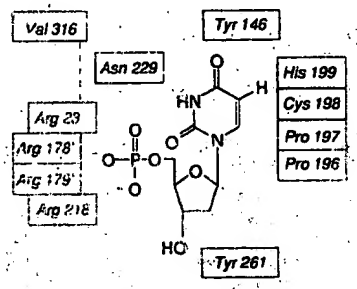
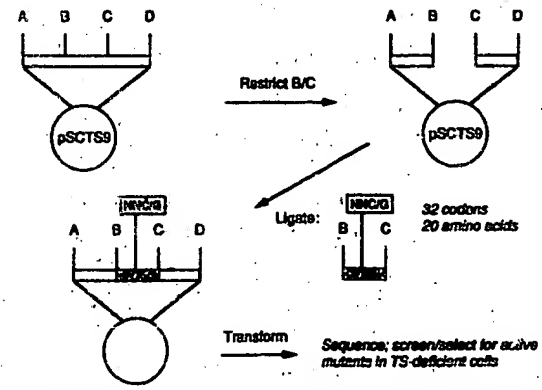


FIG. 1. Amino acid residues of TS targeted for mutagenesis depicted near the sites of interaction with dUMP. - - -, hydrogen bond between Val^{316} and Arg^{23}.



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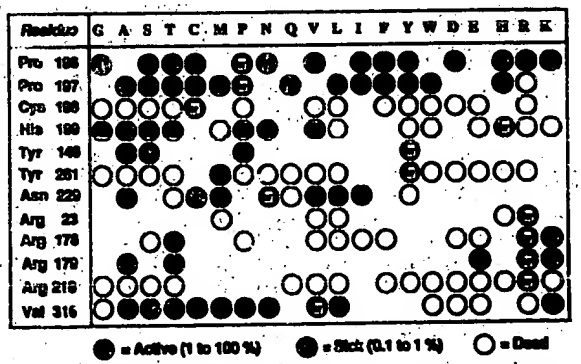

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Tyr^{261} is a completely conserved residue which forms a hydrogen bond to the 3′-hydroxyl of dUMP in the ternary complex. We isolated 14 amino acid mutations, the wild-type Tyr, and the amber codon at position 261 after sequencing 41 clones. Of these, only the Met mutant was active by complementation.

Asn^{229} is completely conserved and lies in the highly conserved, hydrophobic J-helix which forms the core of TS. In the ternary *E. coli* TS·dUMP·CB3717 complex, Asn^{229} interacts with bound dUMP via hydrogen bonds between the γ-oxygen of Asn and NH-3 of dUMP and between γ-NH_2 and O-4. We isolated 9 of the 20 possible amino acid substitutions, the parental Asn, and the amber codon after sequencing 13 clones. Five of these were active by complementation.

The carboxyl terminus of most TS sequences is Val, although Ile, Leu, and Ala have been found. It appears that the carboxyl-terminal residue is important to TS since removal of Val^{316} in *L. casei* TS by carboxypeptidase leads to a loss of folate cofactor binding and catalytic activity (15). Interestingly, on forming TS·dUMP·CB3717, the carboxyl-terminal residues undergo a large movement toward the active site where they approach the folate analog (4). The terminal Val^{316}carboxylate is hydrogen-bonded to the essential Arg^{23} and conserved Trp^{85} which probably stabilize the conformer. We isolated 14 amino acid mutations at position 316, the wild-type Val, and the amber codon after sequencing 29 clones. Of these, nine were active by complementation. As expected, the amber mutant lacking terminal residue 316 was not active by the complementation assay.

SUMMARY

We have described a simple strategy for saturation mutagenesis of any residue of TS and for the rapid identification of mutants which are catalytically active. The targeted codon of a synthetic gene is replaced by a mixture of duplex oligonucleotides which encodes all 20 amino acids. After isolation of individual mutants, a complementation assay using TS-deficient *E. coli* rapidly identifies those mutants with catalytic activity.

The important components of the approach we have used which make it generic include the following. (*a*) A synthetic gene with conveniently placed, unique restriction sites permits mutagenesis at any position at nearly 100% mutagenesis efficiency. (*b*) A mutagenesis vector which also serves as a high expression vector avoids further subcloning. (*c*) A selection system for the desired property permits rapid identification of mutants of interest.

We have made replacement sets of amino acid substitutions at 12 conserved residues that are believed to play roles in structure and function of TS. We isolated some 125 different site-directed mutants, 57 (46%) of which were catalytically active. With the exception of Cys^{198}, Arg^{23}, and Arg^{218}, none of the residues that were mutagenized are strictly essential for activity. All of the active replacement positions had mutants with at least 1% of the activity of wild-type TS, and many have been found with k_{cat} values approaching that of the natural enzyme.[3] Despite the wealth of information available on TS, it would not have been possible to predict the

[3] S. Climie, L. Ruiz-Perez, D. Gonzalez-Pacanowska, P. Prapunwattana, S.-W. Cho, R. Stroud, and D. V. Santi, unpublished data.

range of allowable substitutions, and the results stand as a caution to assigning structure-function roles of a given residue based on one or a few mutants. To explain how TS can tolerate modifications at so many conserved residues of presumed importance, we suggest that there is a high plasticity of the structure of this protein in accommodating function; that is, when a mutation is made that is potentially detrimental to function, the structure may be sufficiently flexible to accommodate the change. Furthermore, it appears that many of the amino acids studied may not simply serve a role in catalysis, or else they would not have survived evolutionary pressure. We are searching for other contributing factors to the conservation of these residues.

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